Volume 336, number 1, 181–183 FEBS 13405 December 1993


# Electron microscopy of two-dimensional crystals of mitochondrial ATP synthase

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Received 15 September 1993

Two-dimensional crystals of the mitochondrial ATP synthase up to 0.4 μm in size were obtained from the detergent-lipid-protein micelles by detergent dialysis. A projected map of the negatively stained crystal was calculated from electron microscopical images by the Fourier-filtering procedure at about 2.8 nm resolution. The unit cell (with not more than two ATP synthase molecules) has the following parameters: $a$ = 13.0 nm, $b$ = 25.6 nm and $\gamma$ = 86°. Two alternative models for the crystal structural organization were suggested, viz. with one or two protein molecules per unit cell.

ATP synthase; Two-dimensional crystal; Electron microscopy; Image processing

## 1. INTRODUCTION

ATP synthases carry out ATP synthesis (hydrolysis) coupled to transmembrane proton transport. The enzymes from different sources were found to be structurally similar. They consist of two main components: the integral membrane portion $F_0$ and the membrane-associated portion $F_1$ ($F_1$-ATPase). $F_1$ contains all catalytic and nucleotide-binding sites and $F_0$ apparently forms a proton channel (reviews in [1,2]). Mitochondrial ATP synthase consists of at least twelve subunits: $\alpha$, $\beta$, $\gamma$, $\delta$ and $\varepsilon$ in $F_1$ (in 3:3:1:1:1 stoichiometry) and a, b, c, d, OSCP (oligomycin sensitivity-conferring protein), $F_6$ and $A_6L$ in $F_0$ [3,4].

According to electron microscopy [5,6] and X-ray analysis [7], the $F_1$-ATPase has a bilayer structure: $3\alpha$ and $3\beta$ subunits are located on two planes and connected by 3-fold rotational symmetry, with the central space occupied by seventh protein mass ($\gamma$, $\delta$ and $\varepsilon$ subunits).

The $F_0F_1$ spatial structure was not sufficiently studied. According to electron microscopy the $F_1$-ATPase is connected with the $F_0$ part by the stalk about 4.2 nm in length and 3–4 nm in width, actually being outside the membrane [8,9]. The dimensions of the hydrophobic $F_0$ component embedded in the membrane are 6–9 nm [8,9] to 6–12 nm [10], with its small part locating outside the lipid bilayer.

The structural organization of membrane proteins can be studied by electron microscopy of two-dimensional crystals followed by the Fourier image processing [11]. The projected structure of ATP synthase crystals was determinated here by those methods at about 2.8 nm resolution.

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## 2. MATERIALS AND METHODS

Bovine heart mitochondria were isolated according to Crane and Green [12]. Submitochondrial particles were prepared by the Racker and Fesseden method [13]. The ATP synthase was solubilized from the submitochondrial particles with sodium cholate, then extracted by precipitation with ammonium sulfate as described by Serrano et al. [14]. Protein concentrations were estimated by the Bradford method [15]. The ATPase activity was measured as described in [16]. The ATP synthase two-dimensional crystals were obtained from detergent-lipid-protein micelles by detergent dialysis as described in [17].

The preparations were negatively stained by 2% w/v uranyl acetate. The grids were examined in the Jeol EM 100CX-2 electron microscope at magnification of 31,000 and with the accelerating voltage of 80 kV. The magnification was calibrated with catalase crystals (Balzers).

Electron micrographs were preliminarily analysed with laser diffractometer as described in [18]. They were digitized on the Joyce-Loebl MDM-6 automatic microdensitometer, with a step size of 30 μm, that corresponded to about 1 nm in the images. The areas recorded consisted of 256 × 256 arrays of optical density values. Image analysis was carried out on the IBM/AT 286 computer, using programs described in [19].

## 3. RESULTS AND DISCUSSION

Earlier [17], we established that two-dimensional crystals of the ATP synthase were formed within the narrow pH range of 4.5–5.0. The optimum conditions for crystal obtaining should be: concentration of $F_0F_1$ 3 mg/ml; lecithin from soybean/lauryldimethylamine $N$-oxide/$F_0F_1$ (1:1:1 by mass); composition of dialysis me-

dium: 50 mM $CH_3COONa$, pH 4.5, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM $NaH_2PO_4$; time of dialysis: 24 h; 20°C. The ATPase specific activity of the reconstituted ATP synthase was between 5 and 6 $\mu$mol of ATP hydrolyzed/min/mg protein. The preparation exhibited 70–80% oligomycin sensitivity, which demonstrated intactness of the multisubunit enzyme complex.

Typical electron micrograph of the ATP synthase crystal is presented in Fig. 1. The maximum size of the crystals is about 0.4 $\mu$m. The optical diffraction and Fourier analysis of the crystal images showed the resolution to be about 2.8 nm. A unit cell has the following parameters: $a$ = 13.0 nm, $b$ = 25.6 nm, $\gamma$ = 86°. Fig. 2 presents Fourier-filtered image of negatively stained two-dimensional crystals of ATP synthase (projection on the membrane plane). Note that stain-excluding regions corresponding to the protein molecules (negative staining of the protein) form typical bands in the direction of vector $\vec{a}$. It was established that one of the projections of the $F_1$ molecule known as frontal was hexagonal, approx. 12 nm across [6,7] and the side projections were in the shape of rectangle or parallelogram, about 7–7.4 nm in width [7,20]. The band thus consists of the aligned ATP synthase molecules with the $F_1$ parts presumable contacting to each other and with their hexagonal profiles parallel to the membrane surface.

So, the ATP synthase molecule projection on the membrane plane corresponds to a circular region with a diameter of about 12 nm. It contains six maxima of the protein density around the stain-filled cavity, each of the two neighbouring ATP synthase molecules making its own contribution to the two maxima in-between (see Fig. 2). In all probability, the absent of hexagonal symmetry is primarily a result of marked distortions in $F_1$ molecules packing on the membrane plane (it is these distortions that set a resolution limit equal to 2.8 nm). In addition, the frontal side of the $F_1$ may be slightly tilted relative to the membrane plane, resulting in the symmetry disruption.

Comparison of the unit cell dimensions (13 × 25.6 nm) with those of the frontal projection of $F_1$ (12 × 12 nm) led to the conclusion that there was one or two ATP

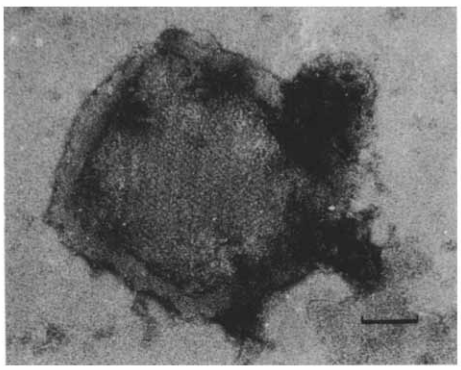

Fig. 1. Electron micrograph of negatively stained two-dimensional crystals of ATP synthase. Scale bar = 0.1 $\mu$m.

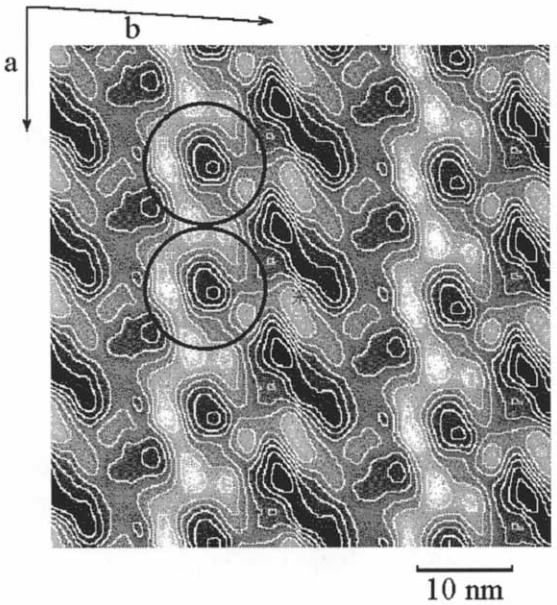


Fig. 2. Fourier-filtered image of negatively stained two-dimensional crystals of ATP synthase (projection on the membrane plane). Light regions are stain-excluding regions (protein), dark regions are stain-filled regions (lipid). Encircled regions show the ATP synthase molecules as seen from the $F_1$ parts. The basic vectors of the unit cell, $a$ and $b$ are depicted by the arrows.

synthase molecules per unit cell. In the first case, the stain-excluding region, interposed between neighbouring protein rows, corresponds to lipid surroundings. In the second case, it corresponds to the integral membrane part $F_0$ of the other ATP synthase molecule with the opposite orientation in the membrane. Therefore, two alternative models for the crystal structural organization may be proposed: with one or two protein molecules per unit cell (see Fig. 3). The first model offers the asymmetric incorporation of the protein molecules into the lipid bilayer: extramembranous portions $F_1$ are located on one side of the crystal membrane plane. According to the second model, the incorporation occurs on each side of the lipid bilayer: there are two ATP synthase molecules with the relative opposite orientation per unit cell.

Noteworthy, according to the first model (see Fig. 3A), the following two types of the projected images are to exist: a projection of the crystal membrane from the $F_1$ parts shown in Fig. 2 and a rear view of the crystal membrane is rather different. Since the images of the latter were not found, the second model should be preferable. Besides, only this model suggests that a regular arrangement of the protein molecules in the crystal is provided by their interactions with each other within a protein row (by way of the $F_1$ parts) and between them (by way of the $F_0$ parts). The protein interaction between the rows occurs only in the second model characterized by the interrow space of about 12.8 nm, twice less as compared with the first model. Such space corre-

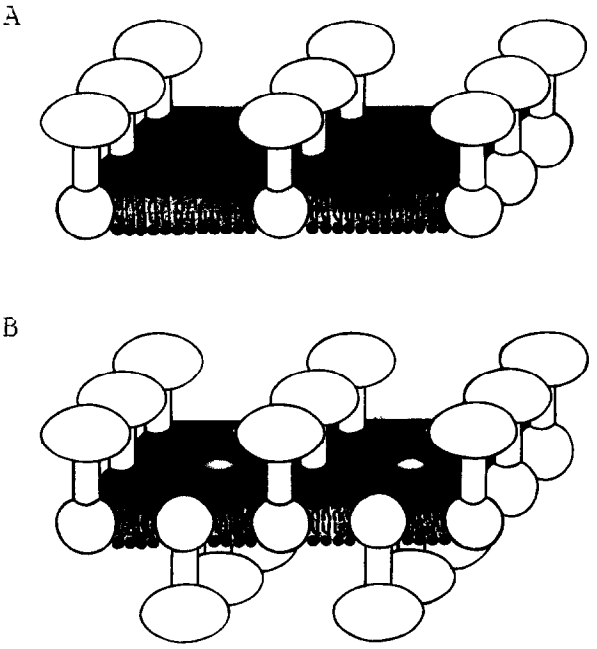


Fig. 3. Two alternative models of the two-dimensional crystal of ATP synthase: with one (A) or with two (B) protein molecules per unit cell.

sponds to the maximum longitudinal size of the $F_0$ (12 nm; see [10]). As for the first model, it suggests that the protein rows interact due to the lipid surroundings, but that is improbable.

The three-dimensional reconstruction is necessary for the final structural determination of the two-dimensional crystals of ATP synthase, that is the aim of our further studies.

*Acknowledgements:* This work was supported by the Ministry of Higher School and Technical Politics of Russian Federation under the National People's Technical Programme (Recent Methods of Bioengineering. Division: Protein Engineering).

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